First publ. in: Pteridines and Related Biogenic Amines in Neurophsychiatry, Pediatrics and Immunology / ed. R. Levine et al. - Grosse Pointe: Lakeshore 1989, pp. 145-150

PURIFICATION AND PARTIAL CHARACTERIZATION OF 6-PYRUVOYL-TETRAHYDROPTERIN REDUCTASE FROM HUMAN LIVER

P. Steinerstauch, Y. Sawada, W. Leimbacher, S. Ghisla, and H.-Ch. Curtius

INTRODUCTION

Reduction of 6-pyruvoyl tetrahydropterin (PPH_4) during biosynthesis of BH_4 can be accomplished by two distinctly different enzymes requiring NADPH. One is sepiapterin reductase (SR) which catalyzes the reduction of both keto functions in the side chain of PPH_4 (4,9,12). However, it seems as if the 1'-keto function is preferentially reduced since 6-lactoyl tetrahydropterin was never observed to be a product of SR (7,9). The second enzyme is 6-pyruvoyl tetrahydropterin reductase (PPH_4R) which is specific for the 2'-carbonyl function (2,6,7,9,13,14). This brings up two important questions: Are both enzymes required for efficient formation of BH_4? If so, which is the reduction sequence of the two functions, i.e., are 6-lactoyl or the isomeric 6-(1'-hydroxy-2'-keto)-propyl tetrahydropterin intermediates? Or, alternatively, are both pathways possible?

In an earlier report (2) we described the preliminary purification and some of the PPH_4R properties. In the meantime we refined the procedure for the purification of this enzyme, investigated several of its properties and obtained polyclonal antibodies. With the present contribution we report on some unpublished studies with PPH_4R. A full report on the subject will be submitted shortly (10).

RESULTS AND DISCUSSION

For obtaining information on the questions outlined above, the determination of the kinetic parameters of PPH_4R and comparison with those of SR are of great importance. A

main problem in this context is the assay procedure. Smith (8,9) devised assays involving the use of purified PPH_4. The instability of the latter has prompted us to investigate the use of a combined assay involving production of PPH_4 from dihydroneopterin triphosphate (NH_2TP) in the presence of pyruvoyl tetrahydropterin synthase (PPH_4S) in situ. Thus 10 µM NH_2TP in 25 µl phosphate buffer, pH 7.0, containing 8 mM $MgCl_2$, were incubated anaerobically at 37°C with 10 mU PPH_4S, as detailed in the legend to Figure 1. The formation of PPH_4 was determined at time intervals by adding 20 µl of a 0.2 M EDTA solution, and subsequent HPLC analysis (electrochemical detection (1)).

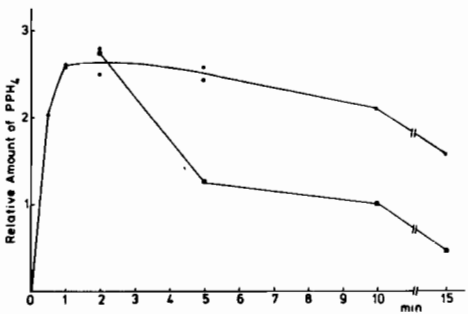

Figure 1:

Time dependent PPH_4 formation -•- and its consumption -•- by PPH_4R. 1 nmole NH_2TP was preincubated with 10 mU of PPH_4S (salmon liver (3)) in the presence of 0.1 M phosphate buffer, pH 7.0, 8 mM $MgCl_2$, 0.25 mM DTE, 10 mM glucose, 12 U glucose oxidase, 4800 U catalase, and 2 mM NADPH, in a total volume of 100 µl. After different incubation times as indicated, 20 µl of a 0.2 M EDTA solution were added to stop the reaction. The decrease of PPH_4 (produced within 1 min) was measured after further incubation with ~14 µU of PPH_4R. The formation of 6-lactoyl tetrahydropterin was stopped after 1 min, 4 min, 9 min, and 14 min, using 20 µl of a 0.25 M NaF solution containing 50 mM DTE.

We found, that under these conditions maximal formation of PPH_4 is observed after 1-2 min (Fig. 1), and that the concentration of the product subsequently decreases with a half time of ≥ 10 min. In our hands this method produces reproducibly PPH_4 of a defined concentration deviating ~10 % from the expected value. When PPH_4R is added at this point in the presence of NADPH, 6-lactoyl tetrahydropterin is formed. After stopping the reduction with 20 µl of a 0.25 M NaF solution the remaining PPH_4 was determined by the use of HPLC and electrochemical detection (1).

With the help of these methods and by systematic variation of the respective substrate concentrations, the K_m and V_{max} values shown in Table 1 were estimated and compared with corresponding values obtained by Smith (9) and Sueoka et al. (11) for SR.

Table 1: Selection of kinetic parameters estimated for PPH_4R and comparison with SR

	PPH_4R	SR
K_m (PPH_4) (µM)	1.8	2.0 a)
K_m (NADPH) (µM)	5.5	1.7 c)
V_{max} (PPH_4) (µmol/min/mg)	1.1	1.0 b)
V_{max} (NADPH) (µmol/min/mg)	0.8	21.7 c)

a,b) data for the kinetic parameters of SR from rat erythrocytes with PPH_4 as substrate were taken from (9); b) V_{max} (PPH_4) is referred to by Smith as "V_{rel}" (9); c) kinetic data for NADPH (11) were determined using sepiapterin as substrate. The values shown for PPH_4R were estimated using an in situ assay as detailed in the text.

The values listed in Table 1 indicate that PPH_4 is reduced with similar efficiency by both enzymes. However, the above values should be taken with due caution since the parameters available for SR refer to the enzyme of sources different from human liver. Furthermore, K_m and

V_{max} values for NADPH and SR were determined using sepiapterin as substrate.

Due to the high instability of SR our attempts to isolate and study human liver enzyme have so far been very disappointing (Zagalak et al., unpublished experiments). It should also be pointed out that the above values might not be relevant for the in vivo system, i.e., in the liver, where compartmentation of the different enzymes and substrates cannot be excluded, and where the concentration of the latter might vary as a consequence of the metabolic processes. In order to gain as much insight as possible into the in vivo conditions, a limited number of selected experiments were carried out using crude extracts of human liver. However, we employed 15 μM PPH_4 and 2 mM NADPH, which should be more than sufficient to saturate both SR and PPH_4R.

Under these conditions the relative activity for SR was 138 mU/g liver (production of 6-(1'-hydroxy-2'-keto)-propyl tetrahydropterin) and 41 mU/g liver for PPH_4R (production of 6-lactoyl tetrahydropterin). These values also support the tentative conclusion arising from the kinetic results that the two enzymes might equally well use PPH_4 as a substrate, i.e., that the biosynthesis might proceed indiscriminately via the two alternative and possibly equivalent ways mentioned in the introduction.

In this context the question whether SR catalyzed isomerization (5) might play a role under in vivo conditions and thus eliminate the need for PPH_4R, cannot be answered by the experiments reported here and might be hard to tackle. The question which intermediate, 6-lactoyl or 6-(1'-hydroxy-2'-keto)-propyl tetrahydropterin might be more efficiently reduced by SR in the last step of the BH_4 biosynthesis is also of importance.

A preliminary characterization of the polyclonal antiserum obtained from BALB/c mice against PPH_4R showed specificity for the protein band corresponding to PPH_4R at a working dilution of 1:2000 on Western blot. Immunoprecipitation experiments using pure PPH_4R as well as liver homogenates are in progress and will be published elsewhere.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation, project no. 3.395-0.86. We are grateful to Dr.

G. Schoedon for helpful assistance with production and characterization of polyclonal antibodies against PPH_4R.
Mailing addresses: Division of Clinical Chemistry, Department of Pediatrics, University of Zürich, Steinwiesstr. 75, CH-8032 Zürich, Switzerland. S. Ghisla, Faculty of Biology, University of Konstanz, D-7750 Konstanz, FRG.

REFERENCES

1 Curtius, H.-Ch., D. Heintel, S. Ghisla, Th. Kuster, W. Leimbacher, A. Niederwieser (1985) Biosynthesis of tetrahydrobiopterin in man. J. Inher. Metab. Dis., 8 (Suppl. 1):28-33.

2 Curtius, H.-Ch., P. Steinerstauch, W. Leimbacher, U. Redweik, Sh.-I. Takikawa, S. Ghisla (1987) Purification of 6-pyruvoyl tetrahydropterin 2'-keto reductase from human liver. In: Unconjugated Pterins and Related Biogenic Amines, H.-Ch. Curtius, N. Blau and R.A. Levine (eds.), Walter de Gruyter, Berlin, pp 89-98.

3 Hasler, Th., A. Niederwieser, H.-Ch. Curtius (1987) Purification of 6-pyruvoyl tetrahydropterin synthase from salmon liver. In: Unconjugated Pterins and Related Biogenic Amines, H.-Ch. Curtius, N. Blau and R.A. Levine (eds.), Walter de Gruyter, Berlin, pp 81-87.

4 Katoh, S., T. Sueoka (1984) Sepiapterin reductase exhibits a NADPH-dependent dicarbonyl reductase activity. Biochim. Biophys. Res. Commun., 118:859-866.

5 Katoh, S., T. Sueoka (1987) Isomerization of 6-lactoyl tetrahydropterin by sepiapterin reductase. J. Biochem., 101:275-278.

6 Masada, M., M. Akino, T. Sueoka, S. Katoh (1985) Dyspropterin, an intermediate formed from dihydroneopterin triphosphate in the biosynthetic pathway of tetrahydrobiopterin. Biochim. Biophys. Acta, 840:235-244.

7 Milstien, S., S. Kaufman (1985) Biosynthesis of tetrahydrobiopterin: conversion of dihydroneopterin triphosphate to tetrahydropterin intermediates. Biochem. Biophys. Res. Commun., 128:1099-1107.

8 Smith, G.K., C.A. Nichol (1986) Synthesis, utilization, and structure of the tetrahydropterin intermediates in the bovine adrenal medullary de novo biosynthesis of tetrahydrobiopterin. J. Biol. Chem., 261:2725-2737.

9 Smith, G.K. (1987) On the role of sepiapterin reductase in the biosynthesis of tetrahydrobiopterin. Arch. Biochem. Biophys., 255:254-266.

10 Steinerstauch et al., in preparation.
11 Sueoka, T., S. Katoh (1982) Purification and characterization of sepiapterin reductase from rat erythrocytes. Biochim. Biophys. Acta, 717:265-271.
12 Sueoka, T., S. Katoh (1985) Carbonyl reductase activity of sepiapterin reductase from rat erythrocytes. Biochim. Biophys. Acta, 843:193-198.
13 Switchenko, A.C., J.P. Primus, G.M. Brown (1984) Intermediates in the enzymatic synthesis of tetrahydrobiopterin in Drosophila melanogaster. Biochem. Biophys. Res. Commun., 120:754-760.
14 Takikawa, Sh.-I., H.-Ch. Curtius, U. Redweik, W. Leimbacher, S. Ghisla (1986) Purification and characterization of 6-pyruvoyl-tetrahydropterin synthase from human liver. Eur. J. Biochem., 161:295-302.